FATTY ACID COMPOSITION OF COMPONENT LIPIDES FROM HUMAN PLASMA AND ATHEROMAS*

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The mounting evidence within the past few years that suggests the important role which dietary fats may play in renal-cardiovascular diseases has stimulated an unprecedented interest in almost every aspect of lipide metabolism, as indicated by the great increase in publications in this field. Unfortunately, owing to lack of satisfactory micro- or semimicro-methods for separating lipide components and determining their fatty acid composition, little has been published on the effect of dietary fatty acids on the fatty acid composition of the various body tissue lipides, although the need for these comprehensive studies has certainly been recognized.

Considerable attention is being given to the development of improved methods for the purpose, and recent advancements in lipide fractionation and analysis offer promise of useful application in lipide metabolism research: elution chromatography on silicic acid columns for separating extracted lipides into their components, *i.e.* sterol esters, glycerides, free sterols, and phospholipides (1–3); reverse phase partition chromatography for fractionating mg. quantities of fatty acids (4); gas liquid partition chromatography for analytical separation and determination of mg. quantities of fatty acids (5); and a spectrophotometric micromethod for determining polyunsaturated fatty acids in mg. sized samples (6).

These methods should still be considered in the developmental state, but, at least, attempts are being made to improve them. For example, Lipsky *et al.* (7) recently reported a modification of Borgström's silicic acid column technique, Crombie *et al.* (8) extended the reverse phase partition technique of Howard and Martin (4), apparatus for gas-liquid chromatography and techniques for its application to fatty acid analysis are still undergoing more or less continuous change and refinement; similarly, extensions and refinements have recently been proposed for the

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spectrophotometric micromethod (9, 10). Although it seems probable that gas-liquid chromatography may ultimately be the method of choice for determination of fatty acids, the present status of its development leaves much to be desired as a routine quantitative method for determining unsaturated fatty acid components. Indeed, there is great need for more extensive experience in different laboratories with all of these more recent methods.

Very little has been published on the application of the recent advances in methodology for the separation of lipides of body tissues into their components and for the determination of fatty acid composition of each. Early studies on the nature of the fatty acids of cholesterol esters and other component lipides were limited to determination of the degree of unsaturation by means of iodine values (11–13). Recently, alkali isomerization spectrophotometric methods (14, 15) have been employed to determine the polyunsaturated acids of total lipides of human plasma. Mukherjee *et al.* (16) employed silicic acid chromatography to separate the component lipides of rat blood and a spectrophotometric method to determine the fatty acid composition of each. Freeman *et al.* (17) described a chromatographic method used in conjunction with infrared spectrophotometry to determine the proportions of lipide components in serum lipides, but did not report fatty acid analysis.

The present paper describes the separation of component lipides of human plasma and atheromas by means of silicic acid chromatography, and the determination of fatty acid composition of the component lipides by means of ultraviolet spectrophotometry and iodine numbers.

EXPERIMENTAL

Source of Tissue—The plasma was obtained by centrifuging whole blood from two healthy young male medical students (12 hours without food) for 1 hour at 2000 r.p.m. The plasma was pipetted from the cells and was first extracted with Delsal (18) solvent (4:1 methylal-methanol), and then the coagulated solids were given a final extraction with peroxide-free ethyl ether. After removal of the solvent, the crude lipide residues were reextracted with redistilled petroleum ether (b.p. 60–70°) and combined. From 415 ml. of plasma (corrected for added anticoagulant—acid-citrate-dextrose solution) 2.052 gm. of petroleum ether-soluble lipides were obtained.

The lipides from atheromas were obtained as follows. Atheromatous plaques, some ulcerated, were excised from human aorta taken at autopsy and extracted first with several portions of Delsal solvent and with ethyl ether. Then the tissue was finely shredded and reextracted by triturating in a mortar with successive portions of ethyl ether. After removal of

the solvents, the lipide residues were reextracted with petroleum ether (b.p. 60–70°) and combined. From 25 gm. of moist tissue, 4.35 gm. of petroleum ether-soluble lipides were obtained.

Solvents—All solvents used in any of the operations reported in this paper were carefully redistilled through a 24 inch Vigreux column before use. The ethyl ether employed as eluting solvent was treated with sodium and then distilled.

Separation of Lipides on Silicic Acid Column—An apparatus similar in design to that described in previous work (19, 20) for operation under slight but constant pressure of nitrogen (1 to 2 cm. of Hg above atmosphere) was used for separation of lipides. The glass column (21 × 300 mm.) was packed by introducing a mixture of 22.0 gm. of silicic acid[1]-filter aid[2] (80:20) in 100 ml. of petroleum ether (b.p. 35–60°). This mixture had been heated to boiling in a beaker, with stirring, for 5 minutes. During addition, slow stirring with a long thin stainless steel rod inserted in the column aided in uniformly packing the adsorbent as it settled by gravity. The adsorbent, when settled in the column, was about 190 mm. in height. The incorporation of a fraction cutter permitted collection of measured volumes of eluate under nitrogen.

About 100 mg. of weighed lipide in 10 ml. of petroleum ether were added by way of a separatory funnel connected to the top of the column. This was quantitatively washed down into the column with a wash of 10 ml. of the solvent, care being taken to keep the solvent level above the top of the adsorbent at all times. The first eluting solvent, 350 ml. of petroleum ether containing 1 per cent ethyl ether by volume, was then added to the separatory funnel, and the flow rate was adjusted to keep a constant level of several inches of liquid above the adsorbent. The rate of percolation about 150 ml. per hour was adjusted by increasing or decreasing the nitrogen pressure on the system. The order of elution, the same as that reported by others, was as follows: (1) hydrocarbons, 50 ml. of petroleum ether (+ 1 per cent ethyl ether); (2) sterol esters, 300 ml. of petroleum ether (+ 1 per cent ethyl ether); (3) glycerides, 300 ml. of petroleum ether (+ 4 per cent ethyl ether); (4) sterols + free acids, 350 ml. of petroleum ether (+ 8 per cent ethyl ether); and (5) phospholipides, 250 ml. of 25:75 methanol-ether followed by 250 ml. of 50:50 methanol-ether.

The separation of the plasma and atheroma lipides was similar except that no hydrocarbons were obtained from the atheroma lipides, and some

[1] Mallinckrodt's analytical grade (100 mesh), suitable for chromatographic analysis by the method of Ramsey and Patterson.

[2] Hyflo Super-Cel filter aid. (The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.)

of the phospholipides of the plasma were so strongly retained on the column that not all were removed. It was found more quantitative and practical to isolate them independently from the total lipides by precipitation with acetone and magnesium chloride, as described by Lipsky *et al.* (7).

Treatment and Analysis of Eluted Fractions—The solvent was removed from the eluted fractions under reduced pressure, and the lipide residues were transferred quantitatively to weighed (tared) 50 ml. round bottomed flasks with petroleum ether. This solvent was then removed, and the flask and contents were brought to dryness by the application of a vacuum and release to atmospheric pressure with nitrogen until constant weight was obtained. The fractions were saponified under nitrogen, the unsaponifiable material was extracted, and the fatty acids were recovered by quantitative extraction after acidification. The weighed fatty acids were made up to a standard volume with petroleum ether (b.p. 60–70°). Aliquots were taken for determination of polyunsaturated acids and iodine values.

Polyunsaturated Acids—The polyunsaturated acids were determined by a modification of the method described by Herb and Riemenschneider (6). Aliquots of the solution of weighed fatty acids obtained from the column separation (2.0 to 7.0 mg.) were added directly to the isomerization tube, and the solvent was removed under a stream of nitrogen and by warming. 2 gm. of 21 per cent KOH-glycol reagent were then added, and the sample was isomerized for 16 minutes at the usual 180°. The tubes were not removed from the bath during the period they were being shaken. (This modified procedure was tested along with the original procedure on numerous samples of different fats within the sample weight range of 1.0 to 7.0 mg., and the results showed good agreement.)

Iodine Value—Aliquots of the solution of weighed fatty acids representing 2.0 to 5.0 mg. of sample were added to 50 ml. glass-stoppered flasks. The solvent was removed under a stream of nitrogen and by warming. Chloroform, 0.4 ml., was added from a hypodermic syringe in such a manner as to wash down the wall of the flask. Exactly 1.0 ml. of 0.2 N Wijs solution was added with a micropipette. The flasks were then stoppered after the stoppers were slightly moistened with potassium iodide solution, and were stored in a dark cabinet for 20 minutes. After this reaction time, the stoppers were loosened enough to permit washing by dropwise addition of 0.4 ml. of 15 per cent KI solution to the flask, followed by the addition of about 4 ml. of distilled water. The iodine was titrated with 0.01 N sodium thiosulfate solution, with 4 to 8 drops of 1 per cent starch solution added as indicator.

RESULTS AND DISCUSSION

The separation of the sterol ester and glyceride fractions on the silicic acid column was essentially quantitative. Analyses of sterol ester frac-

tions from a number of experiments indicated that the sterol content of the ester fractions was from 59 to 62 per cent (theoretical recovery for cholesterol oleate, 59.5 per cent). Recovery of fatty acids from the saponified fractions was about 95 to 96 per cent of the theoretical. The glyceride fractions contained only minor amounts of sterol (1 to 5 per cent), which were removed after saponification and before recovery of the fatty acids. The free sterol and the free acids were eluted together in the same fraction. This fraction from the atheromas contained almost 97 per cent sterol, which had a melting point of 142° (micro melting point). The plasma sterol-acid fraction was usually about 80 per cent sterol. The acid portion of the fraction was confirmed by titrating with standard alkali by a microtechnique. These acids, however, were not isolated. The phospholipide

TABLE I

Component Lipides of Atheroma and Plasma

	Atheroma		Plasma	
	A	B	A	B
	per cent	*per cent*	*per cent*	*per cent*
Hydrocarbon			3.6	2.2
Cholesterol esters	27.8	28.1	39.3	41.3
Glycerides	20.4	18.4	14.3	13.8
Sterols	39.5	38.4	8.3	8.3
Fatty acids	Trace	Trace	2.3	2.2
Phospholipides	13.3	14.1	32.2	32.2

A and B represent duplicate determinations.

content of plasma lipides was obtained independently of the column separation. The small fraction isolated from the plasma before the cholesterol esters was identified by infrared analysis as hydrocarbon. The test for sterol in this fraction was negative. Table I summarizes the lipide composition of atheromas and plasma. Analyses for free and total cholesterol on the extracts (plasma-total cholesterol 31.5 per cent, free cholesterol 8.6 per cent; atheromas-total cholesterol 55.7 per cent, free cholesterol 39.5 per cent) agree very well with those derived from the fractionation data. The free sterols of the atheromas amounted to 70 per cent of the total sterols present, and the free sterols of the plasma were about 26 per cent of the total sterols of that tissue. The phospholipide content of the plasma was almost twice that of the atheromas, and the ratio of phospholipide to cholesterol was also much greater in the plasma than in the atheromas.

The fatty acid compositions of the fractions separated from atheromas and plasma lipides are compared in Tables II, III, and IV. The plasma fractions were almost twice as high in polyunsaturated acids as the cor-

responding fractions from atheromas. Over half of the polyunsaturated acids of both tissues was present as sterol esters. The oleic acid content of the fatty acids of cholesterol esters from atheromas was more than double

TABLE II

*Fatty Acid Composition of Sterol Esters**

Acid	Atheromas (iodine No. 134.0)	Plasma (iodine No. 142.7)
	per cent	*per cent*
Dienoic	23.62†	47.46‡
Trienoic	1.44	0.73
Tetraenoic	7.01	8.00
Pentaenoic	1.27	0.95
Hexaenoic	0.99	0.69
Oleic	61.50	23.49
Saturated	4.17	18.68

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 2.28 per cent conjugated diene.

‡ The value includes 1.02 per cent conjugated diene.

TABLE III

*Fatty Acid Composition of Glycerides**

Acid	Atheromas (iodine No. 84.7)	Plasma (iodine No. 78.3)
	per cent	*per cent*
Dienoic	7.03†	14.27‡
Trienoic	0.52	1.34
Tetraenoic	2.26	1.26
Pentaenoic	0.60	0.47
Hexaenoic	0.00	0.54
Oleic	67.41	44.72
Saturated	22.18	37.40

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 1.61 per cent conjugated diene.

‡ The value includes 2.12 per cent conjugated diene.

that from the plasma. It was also considerably greater in the glyceride and phospholipide fatty acids from atheromas. The saturated acids were somewhat higher in the plasma fractions. It is interesting to note that the cholesterol ester fatty acids of the atheromas contained only small amounts of saturated acids. Infrared spectroscopic examination of the fatty acids showed no measurable amount of trans double bonds.

The analyses of the total fatty acids of atheromas and plasma are presented in Table V. They, of course, reflect the fraction data and show

TABLE IV

*Fatty Acid Composition of Phospholipides**

Acid	Atheromas (iodine No. 77.1)	Plasma (iodine No. 101.8)
	per cent	*per cent*
Dienoic	7.21†	14.53‡
Trienoic	2.30	0.97
Tetraenoic	2.57	8.16
Pentaenoic	0.00	1.76
Hexaenoic	0.00	1.42
Oleic	55.70	35.50
Saturated	32.22	37.66

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 2.93 per cent conjugated diene.

‡ The value includes 3.27 per cent conjugated diene.

TABLE V

*Fatty Acid Composition of Total Lipides**

Acid	Atheromas (iodine No. 110.0)	Plasma (iodine No. 113.0)
	per cent	*per cent*
Dienoic	15.99†	25.21‡
Trienoic	1.07	1.45
Tetraenoic	5.70	6.70
Pentaenoic	1.29	1.26
Hexaenoic	1.09	1.46
Oleic	54.40	32.51
Saturated	20.46	31.41

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 1.68 per cent conjugated diene.

‡ The value includes 1.31 per cent conjugated diene.

the atheromas to be high in oleic acid but appreciably lower in both the saturated and polyunsaturated acids.

The fatty acid content of the fractions is of particular interest in the light of certain suggestions which have been made concerning the possible effect of saturated *versus* unsaturated acids on cholesterol atheromatous deposits. It has been theorized that a high saturated acid content of the diet may cause a preponderance of cholesterol esters of saturated acids in the blood.

These saturated acid sterol esters have a higher melting range and are less soluble and possibly less compatible in the blood than are the unsaturated esters. Thus, they may have a greater tendency to deposit in the coronary walls. If this is true, the cholesterol esters of the atheromas should have a higher saturated acid content than those of normal plasma. The results obtained do not support this idea. However, the difference in content of the various unsaturated acids warrants further investigation of the relation between plasma and atheromatous lipide compositions.

SUMMARY

Elution chromatography on silicic acid columns has been employed for the separation of tissue lipides into their component sterol esters, glycerides, free sterol-fatty acids, and phospholipides. A study was also made of the application of the spectrophotometric method for analysis of fatty acid composition of the lipide fractions. These procedures were successfully applied to the separation and analysis of lipides from human plasma and atheromas. The polyunsaturated acid content of the lipides from plasma was much greater than that for the corresponding lipides from atheromas, whereas the oleic acid content was much greater in the atheroma lipides. The saturated acid content of the cholesterol esters and glycerides from atheromas was considerably less than in corresponding fractions from plasma.

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